REMARKS

Status of the Claims

Claims 18-44 were pending in this application upon receipt of the Office Action. Applicant has amended claims 18, 20, 25-28, 30, 35-37, 42 and 44. Applicant has canceled claims 22, 23, 32, 33, 41 and 43. Applicant has added new claims 45-64. Support for the amendments to these claims can be found in the specification in general and specifically in paragraphs 0009-0016 of the published application, US 2001/0024803 A1.

Objections to the Specification

The Examiner objected to the arrangement of the specification for lacking specific section headings. Applicant acknowledges that 37 C.F.R. § 1.77 details the preferred arrangement of an application. The language of that section, however, does not require those headings.

Applicant reiterates that the instant application is based on Application No. 100 13 337.0, which was originally structured for filing in the German patent office without headings. Applicant demurs from adding section headings because characterization of certain parts of the specification as "Field of the Invention" or "Background of the Invention" can lead to inadvertent admissions against interest when an application was not originally structured to accommodate them.

For example, an application originally structured without headings may interweave discussions of the prior art with comparisons to the claimed invention, upon which the inventors seek to rely for support. If such a segment of the specification were to be labeled "Background of the Invention" or "Description of the Related Art," an

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applicant might be prevented from relying on it in support of the claims. More importantly, these headings are merely preferred, and are not required by statute. Therefore, to avoid possible error, Applicant respectfully requests that this objection be withdrawn.

Applicant has amended the specification to insert the description of the figures at page 4 at line 21 and has deleted the previous description of the figures at page 13. Accordingly, Applicant requests that Examiner's objection to the location of the description of the figures be withdrawn.

Rejection of Claims 18-44 under 35 U.S.C. § 112, second paragraph

The Office rejects claims 18-44 as being indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. Applicant has amended the claims, by making explicit what was implicit in the claims, as follows.

Claims 18 and 28 have been amended to recite the measurement of macroaggregates of blood platelets and to clarify that the sample being measured contains blood platelets. Claims 18 and 28 have also been amended to substitute the word "activator" for "reaction mixture ingredients" and to make clear that the activators induce platelet aggregation.

Claims 20 and 30 have been amended to correct the lack of antecedent basis.

Claim 28 has been amended to clarify how the method compares the first aggregation measurement to the second aggregation measurement.

Claim 43 has been canceled.

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Claim 44 has been amended to clarify that the mixing in the second reaction phase is adjusted to a lower intensity than the reaction in the first reaction phase.

It is believed that these changes overcome the indefiniteness rejection of claims 18-44.

Rejection of claims 18-21, 24-31, 34-37, and 40-41 under 35 U.S.C. § 102(b): Longmire

Examiner rejects claims 18-21, 24-31, 34-37, and 40-41 as anticipated by Longmire et al ("Longmire"; *Long-range Interactions in Mammalian Platelet Aggregation*, Biophys. J., 58:299-307 (1995)). Applicant traverses this rejection and provides the following remarks.

Applicant first notes that a *prima facie* case of anticipation is established only if the reference expressly or inherently teaches every element or limitation of the claim. M.P.E.P. § 2131. All of the claims in this application relate to a method for measuring macroaggregation of blood platelets after (1) mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and (2) mixing the reaction less vigorously or not at all in a second reaction phase. Longmire measures only microaggregation, rather than macroaggregation, in an unstirred phase after an initial stirring phase of 0.5 seconds. *See* Longmire at pages 301, right column, first full paragraph ("Time courses for platelet recruitment into microaggregates . . . of human and rabbit PRP stirred with 10 μm ADP for 0.5 s, and then left unstirred for Brownian-diffusion-dependent aggregation are shown in Fig. 1.") and 306, left column, first full paragraph ("[I]n fact, the largest aggregates in the nonstirred studies are triplets.").

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Such microaggregates are not measurable by optical or electromagnetic means. *See* paragraph 0009 of the published specification and page 88 of Chapter Four, Platelet Aggregation in Platelets and Their Factors, attached as Appendix A. Although Longmire mentions macroaggregation, it is not mentioned in the context of a method for measuring macroaggregation without the requirement of continuous stirring. *See* Longmire at pages 301, left column, third full paragraph and 304, left column, first full paragraph. Accordingly, Longmire does not anticipate the present invention, as macroaggregation of blood platelets is not measured after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase.

Since the reference does not expressly or inherently teach every element or limitation of the claim, specifically measurement of macroaggregation after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase, the Examiner has failed to provide a *prima facie* case of anticipation, and Applicant requests that the Examiner withdraw this rejection.

Rejection of claims 22-23 and 32-33 under 35 U.S.C. § 103(a): Longmire and Kitek

The Examiner also rejects claims 22-23 and 32-33 as obvious in light of Longmire in combination with Kitek et al ("Kitek"; *Optical Density Variations and Microscopic Observations in the Evaluation of Platelet Shape Change and Microaggregate Formation*, Thromb. Haemost., 44(3):754-758 (1980)). Applicant has canceled claims 22-23 and 32-33. Since new claims 48-49 and 54-55 contain language

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similar to that in previous claims 22-23 and 32-33, Applicant traverses this rejection and provides the following remarks.

A *prima facie* case of obviousness must meet several essential criteria, including that the prior art references must teach or suggest all of the claim limitations, M.P.E.P. § 2142, and that there is some reason, suggestion, or motivation in the prior art to lead one of ordinary skill in the art to combine the teachings of the references in the manner proposed by the Office. *Pro-Mold and Tool Co. v. Great Lakes Plastics, Inc.*, 75 F.3d 1568, 1573 (Fed. Cir. 1996); M.P.E.P. § 2143. The combination of references must also provide a reasonable expectation of success. *In re Dow Chemical Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988). The suggestion or motivation must be found in the prior art, not in the Applicant's disclosure. *Id.* Furthermore, the suggestion to combine the prior art teachings must be clear and particular. *In re Dembiczak,* 175 F.3d 994, 999 (Fed. Cir. 1999). Thus, while a person of ordinary skill in the art may possess the requisite knowledge and ability to modify the prior art, that modification is not obvious unless the prior art suggested the desirability of such a modification. *See In re Gordon*, 733 F.2d 900, 902 (Fed. Cir. 1984).

Claims 48-49 and 54-55 depend from claims 18 and 28 and therefore, incorporate all of the limitations of those claims. As described in the previous section, Longmire does not teach or suggest a method for measuring macroaggregation after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase and Kitek does not correct this deficiency. Since neither Longmire nor Kitek, separately or together, teach or suggest or discuss measuring macroaggregation

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of blood platelets after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase mixing a reaction mixture in a first reaction phase sufficient to induce formation of macroaggregates, the Examiner has failed to provide a *prima facie* case of obviousness, and Applicant requests withdrawal of this rejection.

Rejection of claims 38-39 and 44 under 35 U.S.C. § 103(a): Longmire

The Examiner also rejects claims 38-39 and 44 as obvious in light of Longmire. Applicant traverses this rejection and provides the following remarks.

The Examiner admits that the cited reference Longmire fails to teach sequential stirring and not stirring the platelet samples (claim 39) as well as slowing the stirring down in the second phase rather than completely stopping the stirring (claim 44). The Examiner then states that it would have been obvious for one of skill in the art to make those adjustments to further evaluate Brownian diffusion-controlled platelet collisions. Office action at 5-6. This statement of the Examiner is conclusory and not based on the only reference cited. The Examiner is reminded that while a person of ordinary skill in the art may possess the requisite knowledge and ability to modify the prior art, that modification is not obvious unless the prior art suggested the desirability of such a modification. *See In re Gordon*, 733 F.2d at 902. Longmire does not do so. Moreover, claims 38-39 and 44 are dependent upon claims that require measuring macroaggregation after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase. As discussed above, Longmire also does not teach or

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suggest such a method. Therefore, the Examiner has failed to make a *prima facie* case of obviousness, and the withdrawal of this rejection is requested.

Rejection of claims 42-43 under 35 U.S.C. § 103(a): Longmire and Minamoto

The Examiner also rejects claims 42-43 as obvious in light of Longmire in combination with Minamoto et al ("Minamoto"; *Detection of Platelet Adhesion/Aggregation to Immobilized Ligands on Microbeads by an Aggregometer*, Thromb. Haemost., 76(6):1072-1079 (1996)). Applicant has canceled claims 43. Applicant traverses this rejection and provides the following remarks with respect to claim 42.

Longmire, as discussed above, does not disclose measuring macroaggregation of blood platelets after mixing the reaction mixture in a first phase for a time sufficient to induce formation of macroaggregates and mixing the reaction less vigorously or not at all in a second reaction phase and Minamoto does not cure the deficiencies of Longmire. Moreover, Minamoto describes an assay measuring the interaction between platelets and beads under constant stirring conditions. *See* Minamoto, p. 1073. Therefore, Minamoto would not make obvious the claimed method wherein the reaction mixture in the second phase is not stirred or stirred less vigorously. Longmire and Minamoto together do not teach or suggest the claimed invention. Accordingly, the Examiner has not made a *prima facie* case of obviousness, and Applicant requests withdrawal of this rejection.

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In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: January 21, 2004

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CHAPTER 4

Platelet Aggregation

A. Ruf, M.M. Frojmovic, and H. Patscheke

A. Introduction

The formation of a platelet thrombus plays a crucial role in hemostasis and also in pathomechanisms of several arterial disorders, including stroke and myocardial infarction. The initial step in thrombus formation is the adhesion of platelets onto vascular subendothelial connective tissue exposed upon endothelial injury (see Chap. 3). Collagen and von Willebrand factor (vWF) are important constituents of the subendothelial matrix which mediate adhesion and subsequent activation of the platelets. Platelet activation allows interplatelet contact and the formation of platelet aggregates. In this chapter, the molecular mechanisms mediating platelet aggregation and the tests to assess this platelet function in vitro are summarized. In addition, recent progress in the selective inhibition of platelet aggregation as a therapeutical principle is addressed.

B. Mechanism of Platelet Aggregation

I. The Glycoprotein IIb-IIIa Complex

It is well established that the platelet membrane glycoproteins (GP) IIb and IIIa are essential for platelet aggregation (Kieffer and Phillips 1990; Nurden 1994). The glycoproteins IIb and IIIa form a heterodimeric complex in the platelet membrane which belongs to the protein superfamily of integrins (Hynes 1987; Ruoslahti 1991). Platelets from patients with thrombasthenia lack the GPIIb–IIIa complex or express it in malfunctional forms and are not able to aggregate (George et al. 1990). The complex constitutes a promiscuous receptor for several ligands such as fibrinogen, vWF, fibronectin, vitronectin, and possibly other cytoadhesive proteins (Plow et al. 1985; Haverstick et al. 1985). The complex is exposed on resting platelets in an inactive form and platelet activation is required to transform it into a conformational state which is competent for ligand binding (Sims et al. 1991; Frelinger et al. 1991; Du et al. 1991). Fibrinogen contains distinct amino acid sequences which mediate the binding to GPIIb–IIIa (Hawiger et al. 1982). These include the Arg-Gly-Asp (RGD) sequences in the Aα-chain and a dodecapeptide located at the carboxy terminal γ-chain (Fig. 1). Other adhesive

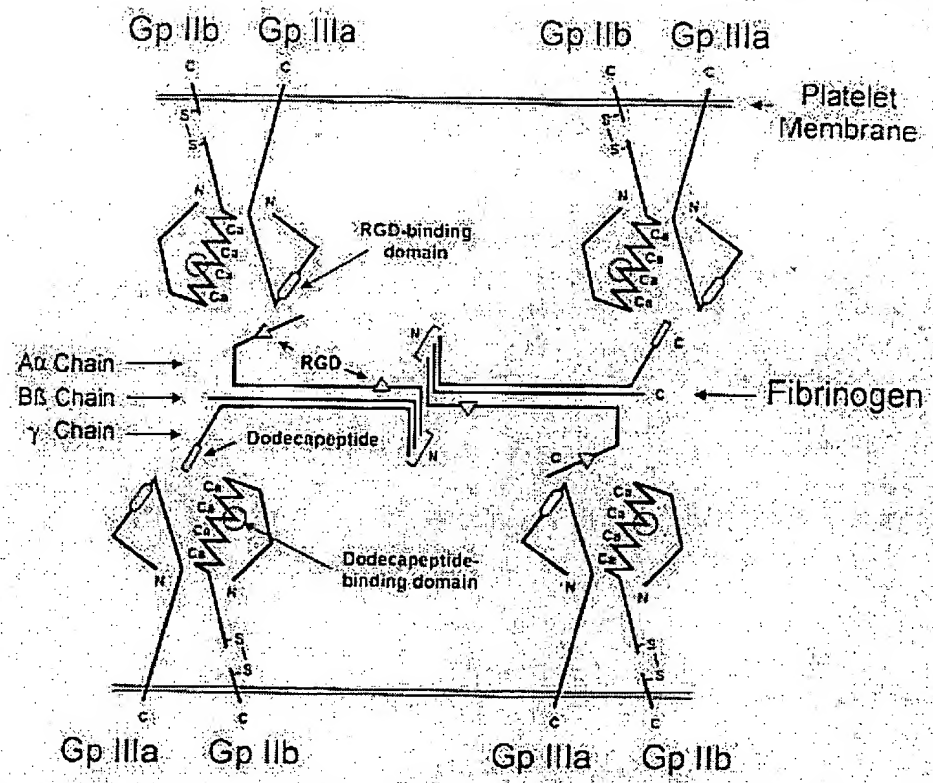

Fig. 1. Fibrinogen as a bridging molecule between two adjacent platelets. *Fibrinogen* has six *GpIIb–IIIa* binding sites: four *RGD* sequences on the *Aα-chains*, and two *dodecapeptides*, one at each carboxy terminus of the *γ-chains*. The RGD-peptide has been cross-linked to GPIIIa (residues 109–171), whereas the dodecapeptide cross-links to GPIIIa (residues 244–314). (Adapted from CHARO et al. 1994)

proteins, such as vWF, bind to the activated GPIIb–IIIa via an RGD motif. On the basis of chemical cross-linking experiments, it was concluded that the primary binding site for RGD peptides is located in GPIIIa (residues 109–171), while the dodecapeptide of fibrinogen cross-links essentially to GpIIb at or close to the second putative Ca^{2+}-binding domain (PLOW et al. 1992; BENNETT 1996).

The peptide GpIIb alpha 300–312 binds, in addition to fibrinogen, also other adhesive glycoproteins such as vitronectin. Furthermore, this peptide inhibits the adhesion of activated platelets to fibrinogen, fibronectin, vitronectin, and vWF (TAYLOR and GARTNER 1992). Thus this peptide may constitute a common recognition site on GPIIb–IIIa for the dodecapeptide and the RGD-containing ligands. Another binding site for fibrinogen seems to be located at residues 211 through 222 of GPIIIa (CHARO et al. 1991). With respect to most of the published studies, it is reasonable to assume that an activation-dependent conformational change in the GPIIb–IIIa complex leads

to the formation of a ligand-binding pocket which is composed of the above peptides.

II. Adhesive Ligands of the GPIIb-IIIa Complex

It is well established that fibrinogen plays an important role in platelet aggregation under low shear conditions (Peerschke 1985; Weiss et al. 1989; Ikeda et al. 1991; Xia and Frojmovic 1994). Human fibrinogen is composed of three pairs of distinct protein chains (α, β, γ) which are linked by various disulfide bonds and arranged in three main domains: one central E and two distal D domains (Doolittle 1984). The amino acid sequence RGD on the Aα-chain (residues α 95–97 and α 572–574) and the dodecapeptide HHLGGAKQAGDV on the carboxy terminus of the γ-chain of fibrinogen (residues γ 400–411) are potential recognition sites for the GPIIb–IIIa complex (Anrieux et al. 1989). Studies using mutated fibrinogens, synthetic peptides, and antibodies against fibrinogen have highlighted the essential role of the dodecapeptide in intact fibrinogen for platelet aggregation (Kloczewiak et al. 1984, 1989; Anrieux et al. 1989; Farrell et al. 1992; Kirschbaum et al. 1992). Recombinant fibrinogens with mutations in RGD sequences supports platelet aggregation as effectively as wild-type plasma fibrinogen. However mutations in the γ-chain dodecapeptide yield variant fibrinogens which are markedly defective in platelet aggregation (Farrell et al. 1992).

Fibrinogen is symmetric and possesses two sets of binding domains at each end; thus, it can form a molecular bridge between two adjacent platelets (Hawiger et al. 1989). Each half of the fibrinogen molecule can potentially bind to two or three receptor sites on the platelet membrane (Fig. 1). The gap between platelets in closely packed aggregates in vivo is 100 to 300 Å wide (Shirasawa and Chandler 1969; Moon et al. 1990). Since the overall length of the fibrinogen molecule is about 475 Å it is likely that the fibrinogen molecule is oriented such that its long axis is parallel to the contact zone of adhering platelets (Fig. 1).

In contrast to the case of low shear conditions where fibrinogen mediates platelet aggregation, at high shear rates, vWF seems to bind to GpIIb–IIa and thereby mediate platelet aggregation and thrombus formation (Fressinaud et al. 1988; Weiss et al. 1989). Platelet aggregation on purified collagen or subendothelium is inhibited by a monoclonal antibody of vWf that selectively blocks vWF binding, but not fibrinogen binding to GPIIb–IIIa (Weiss et al. 1989). Direct evidence that the RGD sequence in vWF is involved was provided by studies in which aggregation was inhibited by monoclonal antibodies that recognize the RGD (residues 1744–1746) sequence in vWF and selectively inhibit vWF binding to GPIIb–IIIa and platelet aggregation at high shear rates (Weiss et al. 1993). Other studies using cone-in-plate viscometers also provided evidence that vWF mediates platelet–platelet interaction at high shear rates whereas fibrinogen does not (Ikeda et al. 1991, 1993; Chow et al. 1992; Ruggeri 1994). These investigations showed that shear rates >4000 s^{-1} in

plasma initiate the binding of multimeric vWF to GPIb, which subsequently induces a transmembrane flux of calcium ions. The increase in intracellular calcium levels leads to platelet activation with an exposure of active GPIIb–IIIa complexes which in turn bind vWF and thereby mediates platelet aggregation. When platelets are extrinsically activated with agonists such as ADP, much lower shear rates (300–1200s^{-1}) will drive platelet aggregation independent of fibrinogen; it is likely that this aggregation is mediated by vWF secreted onto the platelet surface (Goldsmith et al. 1994).

III. Redistribution of the GpIIb-IIIa Complex and Internalization of the Ligands

Stimulation of platelets leads to an increase in the number of GPIIb–IIIa complexes on the platelet surface which may be due to a redistribution from intracellular pools such as the membranes of the dense tubular system and storage organelles (Wencel-Drake et al. 1986; Woods et al. 1986). The binding of fibrinogen induces a clustering of the GpIIb–IIIa complexes which may be caused by cross-linking of neighboring receptors by the multiple binding domains of the fibrinogen molecule in cis position (Isenberg et al. 1987). This clustering is followed by an internalization of fibrinogen; the internalized fibrinogen seems to represent the fraction of irreversibly bound fibrinogen (Peerschke 1995; Wenzel-Drake et al. 1996). Platelets which have internalized fibrinogen lose their ability to aggregate as measured turbidimetrically (Peerschke 1995; Wenzel-Drake et al. 1996). Thus, receptor redistribution and fibrinogen internalization may represent a regulatory mechanism of platelet aggregation. Whether other ligands, such as vWF, can also be internalized is still matter of debate.

C. Platelet Aggregation Testing

1. Sample Preparation

Platelet aggregation testing can be performed with various platelet samples such as whole blood, platelet-rich plasma (PRP), and suspensions of washed and gel-filtered platelets (Mustard et al. 1972; Hutton et al. 1974; Day et al. 1975; Patscheke 1981). The procedures applied during sample preparation may affect the activation state and the functional integrity of the platelets. The factors involved are the blood sampling procedure, the type of anticoagulant used, the pH and temperature during the isolation procedures, and the composition of the platelet suspending media (Mustard and Packham 1970; Zucker 1972; Day et al. 1975; Kinlough-Rathbone et al. 1977a; Packham et al. 1978). Artefactual platelet activation is one of the major problems. This seems mainly to be due to the formation of trace amounts of thrombin during

blood sampling which although not sufficient to initiate fibrin generation may activate platelets (KAPLAN and OWEN 1981; LEVINE et al. 1981). An artifactual preactivation may enhance the aggregation response induced by added agonists or may decrease it in case of a desensitization (KINLOUGH-RATHBONE et al. 1977b; PACKHAM et al. 1973). Whichever effect occurs is dependent on the time and degree of preactivation and is usually unpredictable.

In addition to the procedure of blood collection, the anticoagulant can also affect the function or the activation state of platelets. Heparin has long been recognized as an inducer of platelet activation (EIKA 1972; ZUCKER 1975). Trisodium citrate depresses the Ca^{2+} concentration, and under these conditions platelets liberate arachidonate when they are brought into close contact with one another (DAY et al. 1975; MUSTARD et al. 1975). Arachidonate is converted to thromboxane A_2 (TXA_2) which is a potent platelet agonist. Formation of TXA_2 mediates the release reaction upon stimulation of citrate-anticoagulated PRP with ADP (MUSTARD et al. 1975). It also accounts for the aggregation of platelets when citrated plasma is subjected to centrifugation during washing procedures. This problem does not occur when blood is collected into acid-citrate-dextrose (ASTER and JANDL 1964) which gives a pH of 6.5 or slightly lower and prevents platelet activation and aggregation upon centrifugation.

Platelet-rich plasma obtained by using hirudin as an anticoagulant exhibits minimal activation of platelets and the arachidonate pathway is not activated when the platelets are aggregated by ADP; this is most likely due to the fact that the ionic calcium concentration in this plasma is not depressed (MUSTARD et al. 1975). EDTA can induce the conformational change of platelets and dissociates the GPIIb–IIIa complex at 37°C (GRANT and ZUCKER 1978; FITZGERALD and PHILLIPS 1985). These platelet alterations may be irreversible if platelets are exposed to EDTA for 10–15 min, depending on the temperature. Thus EDTA should not be used as anticoagulant even if it is diluted during the isolating procedures.

When washing or gel-filtrating procedures are to be applied, the buffer used to maintain the pH of the platelet suspending media should not affect platelet function. In particular TRIS should be avoided since it exerts substantial effects on platelet structure and function (LAGES et al. 1975; ZUCKER et al. 1974). The suspending media for platelets should contain some essential constituents, in particular various ions such as Ca^{2+} and a protein such as albumin (DAY et al. 1975; KINLOUGH-RATHBONE et al. 1977a; PACKHAM et al. 1978). Furthermore, glucose should be added to the media since platelets require a source of metabolic energy (KINLOUGH-RATHBONE et al. 1970, 1972). Some ADP may be released from residual red blood cells during prolonged storage of a suspension of isolated platelets. This may cause the platelets to become refractory to ADP. Addition of enzyme systems such as apyrase or creatine phosphate/creatine phosphokinase to the suspending medium will remove this ADP and maintain platelets sensitive to ADP.

II. Optical Aggregometry

The turbidimetric determination of platelet aggregation was introduced by Born (1962a,b). The principal of the method is as follows: light shines through a cuvette which contains a platelet suspension and any transmitted light is detected by a photoelectric cell. Stimulation of the platelets leads to shape change and aggregation. Both phenomena affect the light transmittance of a platelet suspension, although it must be emphasized that only shape change and macroaggregation are measurable. It was found that the microaggregation of platelets which parallels the shape change does not contribute to the initial change in light transmittance until >30%–40% of the singlets have aggregated (Milton and Fromovic 1983; Fromovic et al. 1989). The disc–to–sphere transformation leads to a decrease and the platelet macroaggregation to an increase in light transmittance.

Many, apparently disparate, theories have been proposed to explain the observed turbidity changes related to platelet activation. The effects are mainly due to the different light scattering properties of resting discoid platelets, activated platelets, and platelet aggregates. Latimer predicted by application of light scattering theories that a change in platelet orientation by flow and of their shape leads to change in light transmittance, which he experimentally verified (Latimer et al. 1977). This accounts for the fact that orientation of discoid platelets perpendicular to the incident light beam by stirring the platelet sample leads to an increase in transmittance. Without stirring, discoid platelets become randomly oriented which causes the light scatter to increase and as consequence the light transmission decreases. A suspension of spheroid platelets does not show a stir-dependent change in transmittance since a change in orientation by flow cannot occur. However, if discoid platelets change their shape upon stimulation, light scatter increases and the transmittance decreases. In addition, pseudopod formation moves dry weight away from the center of mass of the cell which causes total light scattering to be decreased; i.e., formation of pseudopods increases light transmittance. However, the decrease in transmittance due to the disc–to–sphere transformation dominates the increase in transmittance caused by pseudopod formation; thus, the net effect is a decrease in transmittance (Latimer et al. 1977).

Indeed, measurement of the change in shape, from an analysis of the mean platelet axial ratio, within the first 10s upon ADP addition, showed that the slope of the initial decrease in light transmission was in good agreement with the theoretical predictions. However, at later times (>40s), pseudopod formation has an important influence on the change in light transmittance (Milton and Fromovic 1983). The extent of the optical effect of shape change strongly depends on the optical geometry of the aggregometer (Latimer et al. 1977; Latimer 1975; Fromovic 1978).

The situation with a suspension of aggregating platelets is even more complex than in suspensions of single plateles. It is a heterogenous system with respect to the size distribution of the particles, i.e., it contains platelets and

aggregates of various sizes. Furthermore, it is a dynamic system with a shift in the size distribution towards large aggregates as long as the aggregation process proceeds. Frojmovic has shown that the law of Lambert–Beer is valid for platelet suspensions at a concentration within the physiological range. Furthermore, he proposed that the law of Lambert–Beer may be rewritten as follows to account for particle aggregation (Frojmovic 1978):

$$E = L\sum_{n=1}^{\infty} N_n K_n$$

where L is the light path length of the cuvette; N_n is the number of n-tuples per unit volume; K_n is the scattering cross-section for a particular n-tuple, where $n = 1$ for singlets of the same size, $n = 2$ for doublets, $n = 3$ for triplets, etc.

Since the scattering cross-section of an n-tuple relative to that of n singlets decreases with increasing n (Chang and Robertson 1976), a particular number of single platelets scatter more light than an aggregate consisting of the corresponding platelet number. Thus the light transmission increases as a consequence of platelet aggregation.

The most widespread parameters used for quantitation of platelet aggregation are the maximal net increase in transmission, the slope of the aggregation curve, and the half-time of the aggregation curve (Frojmovic 1973). All of these parameters are dependent on several factors such as the optical geometry, wavelength, and light path length of the measuring device as well as shape, size distribution, and concentration of the platelets and the flow conditions in the system (Frojmovic 1978). Athough the method has a history of over 30 years there are still major problems regarding standardization between different laboratories. Various types of aggregometer are available, each of which are well defined and stable with respect to the above rheooptical characteristics (Mills 1969; O'Brien 1971; Ten Cate 1972; Born 1972; Sixma 1972; Frojmovic 1978; Latimer 1982). However, these characteristics differ significantly between the different aggregometer types and thus, quantitative results obtained with different types are not comparable.

III. Lumi-aggregometry

Lumi-aggregometers are turbidimeters which are additionally equipped with a photomultiplier rendering them capabable of detecting chemiluminescence (Feinman et al. 1977; Ingerman-Wojenski et al. 1983). Thus, these aggregometers allow the determination of ADP release from platelet dense bodies using the Luciferin/luciferase system. Furthermore, with Lumi-aggregometry it is possible to simultaneously measure the activation of platelets and neutrophils or possibly other phagocytosing white blood cells (Rur et al. 1992). With this application the experimental conditions are adjusted in a way that the increase in light transmission predominantly reflects platelet aggregation and the Luminol-enhanced chemiluminescence reflects the oxidative burst of neutrophils.

Another feature of Lumi-aggregometers is that they can be supplied with an impedance module which can assess platelet aggregation electrically. The principle of electrical aggregometry is as follows (Cardinal and Flower 1980). Two electrodes separated by a small gap are located in the cuvette containing a platelet sample. The electrodes become coated with a monolayer of platelets, and if no platelet agonists are added no further interaction with platelets and the electrodes occurs. Thus, in unstimulated platelet samples the conductance between the two electrodes is constant after the initial coating of the electrodes by platelets. When the platelets are stimulated the electrodes become further coated with aggregating platelets which impair the conductance between the two electrodes. Hence, an increase in impedance can be recorded as a consequence of platelet aggregation.

IV. Determination of Platelet Aggregation by Particle Counting

The concentration of single, free platelets and total particles (platelet doublets, triplets, etc.) decreases as a consequence of platelet aggregation. Thus aggregation can be determined by measuring the concentration of single platelets and/or total particles before and after stimulation of platelet aggregation (Lumley and Humphrey 1981; Gear 1982; Frojmovic et al. 1989). The aggregation can be quantified as the fraction (PA) of platelets which were incorporated into aggregates:

$$PA = 1 - N_t / N_0$$

where N_0 is the concentration of platelets before aggregation and N_t the platelet concentration upon stimulation of aggregation at a time point t. Measurements by electronic particle counters may record platelet aggregates consisting of up to four platelets as "singlets" depending on the platelet size. However, comparisons with microaggregation determined by tedious microscopic analysis of truly singlet platelets have shown comparable sensitivities for the two methods (Frojmovic et al. 1989). Both total particle count and "singlets" can also be determined with highly sophisticated flow cytometers, using the technique first described for similar studies of neutrophil aggregation (Rochon and Frojmovic 1993).

V. Potential and Limitations of Platelet Aggregation Testing

Platelet aggregation testing is still the most often applied functional assay of platelets. It has been used for the characterization of various platelet agonists as well as platelet inhibitors in experimental and clinical research settings. Furthermore, it is an established screening tool for the diagnosis of thrombocytopathies. However, it has to be noted that the use of aggregation testing for assessing the actual in vivo function of platelets is rather limited. This is due to two major problems. Firstly, it is impossible to preserve the in vivo activation state of platelets during sample preparation (see Sect. C.I in

this chapter). The second problem is the standardization of the aggregation testing with respect to the different size distributions of platelets from different donors. It is well known that the rate of aggregation is dependent on the number of platelet collisions in a platelet sample. The collision frequency of particles in suspension is dependent not only on the stirring rate and the particle concentration but also on the particle volume (Manley and Mason 1952). Large platelets collide more frequently than small ones; thus, large platelets may undergo an enhanced aggregation although no functional differences to small platelets may exist. Thus, if a comparison is to be made and differences in platelet aggregation between two different donors are to be detected, an adjustment for different size distributions is required. To compensate for different size distributions of platelets it was suggested by Holme and Murphy (1981) to adjust the extinction of the test and the control platelet sample prior to turbidimetric measurements of aggregation. The rationale for this type of calibration is the fact that the extinction of a platelet sample prior to aggregation reflects the mass and the relative refractive index of the platelets. However, if the extinctions are adjusted, different platelet contentrations between test and control samples have to be accepted.

Among the different methods for aggregation testing turbidimetry is the most widespread application used. There are fundamental differences between the different methods with respect to the material which can be analyzed and the information which can be obtained (Table 1; Frojmovic et al. 1989). In contrast to electrical aggregometry and particle counting, turbidimetry can assess platelet aggregation only in suspensions of isolated platelets such as PRP or washed or gel-filtered platelets and not in whole blood. Turbi-

Table 1. Characteristics of aggregation testing methods

	Particle counting	Optical/Lumi-aggregometry
Advantages	– Applicable to all platelet samples, i.e., whole blood, PRP, washed or gel-filtered platelets – Extremely sensitive for early stages of platelet aggregation, i.e., formation of small aggregates	– Continuous measurements, i.e., kinetic information available – Sensitive for late stages of platelet aggregation, i.e., formation of large aggregates – Simultaneous measurement of other parameters of platelet activation such as shape change or release
Disadvantages	– Insensitive for late stages of platelet aggregation, i.e., formation of large aggregates – Discontinuous measurement	– Insensitive for early stages of platelet aggregation, i.e., formation of small aggregates – Applicable only to samples of isolated platelets, i.e., PRP etc., except for Lumi-aggregometers with impedance modules

dimetric and electrical aggregometry yield kinetic information on the aggregation process. In particular it can easily be seen whether the process is reversible or not. With particle counting this information can only be obtained when samples are taken and measured at different time intervals. Furthermore, counting single platelets is very sensitive for detecting platelet aggregation at early stages of aggregation during which the concentrations of platelets rapidly decreases and only small aggregates are formed; it is completely insensitive at later stages at which mainly the size of the aggregates increases. The opposite is true for optical aggregometry which is very insensitive for the detection of small aggregates composed of few platelets (Milton and Frojmovic 1983). This difference in sensitivity is the reason why, for a given agonist, different concentration–response relationships are obtained when the aggregation is measured by either particle counting or turbidimetry and electrical aggregometry. Therefore, EC_{50} values, i.e., the concentration of agonist producing 50% aggregation, will be much lower with particle counting than with electrical and optical aggregometry. On the other hand, optical and probably also electrical aggregometry is more sensitive to an inhibition of platelet aggregation than particle counting (Frojmovic et al. 1933; Pedvis et al. 1988).

D. Inhibition of Platelet Aggregation as a Therapeutic Principle

GpIIb–IIIa is an attractive therapeutical target for several reasons. It mediates platelet aggregation induced by all physiological platelet agonists. Thus, in contrast to other antiplatelet drugs such as aspirin or ticlopidine, GPIIb–IIIa antagonists are independent of the platelet agonists. Furthermore, GpIIb–IIIa antagonists do not abolish platelet adhesion and thereby minimize the risk of severe clinical bleedings.

The compounds which currently are in development can be classified as follows: (Cook et al. 1994; Cox et al. 1994; Coller et al. 1995; Leerkovits et al. 1995):

1. Antibody fragments, e.g., c7E3 Fab (abciximab, ReoPro; Centocor)
2. Cyclic peptides, e.g., Integrelin (COR Therapeutics)
3. Peptidomimetics, e.g., Ro 44-9883 (Hofman-La Roche); MK-383 (Merck)
4. Orally active compounds, e.g., SC54684 (Searle); Ro 43-8857 (Hofmann-LaRoche); GR144053 (Glaxo); DMP728 (DuPont-Merck)

Disintegrins are not in development due to their antigenicity. Except for the c7E3 Fab, a chimeric recominant Fab version of 7E3 (a murine antibody against GpIIb–IIIa; Coller 1985) the other agents are based on the RGD sequence. They virtually all retain the basic charge relations of the RGD sequence yielding a positive and a negative charge which are separated approximately by 10–20 Å. The various GpIIb–IIIa inhibitors differ from each other with respect to their receptor specificity, pharmacodynamics, and phar-

macokinetics (Cook et al. 1994; Cox et al. 1994; Coller et al. 1995; Lefkovits et al. 1995). The c7E3 Fab (abciximab, ReoPro®) was the first compound to be approved for patients undergoing high-risk angioplasty in the US and in several European and Scandinavian countries. All of these agents are currently under further clinical investigation and the results of the Phase-II and Phase-III studies so far are promising. It is likely that GpIIb–IIIa therapy will prove to be beneficial in thrombotic disorders other than angioplasty (Coller et al. 1995; Lefkovits et al. 1995).

E. Conclusions

Platelet aggregation plays an essential role in thrombosis and hemostasis. The process is mediated by a GpIIb–IIIa heterodimer complex in the platelet membrane which acts as a promiscuous receptor for several ligands such as fibrinogen, vWF, fibronectin, and vitronectin. For the interaction with soluble ligands an activation of the platelets is required which converts the GpIIb–IIIa complex into a conformational state competent for ligand binding. Under low shear conditions fibrinogen is the relevant ligand of GPIIb–IIIa whereas under high shear conditions vWF seems to mediate platelet aggregation. For fibrinogen binding a dodecapeptide on it's γ-chain is essential whereas the binding of other cytoadhesive proteins such as vWF is mediated by an RGD motif. Since a blockade of GpIIb–IIIa inhibits the induction of platelet aggregation by all physiological agonists, GpIIb–IIIa is a promising target for antithrombotic therapies. Several types of GpIIb–IIIa inhibitors are under clinical development and the results of the clinical studies so far are all promising.

Platelet aggregation testing is one of the major functional assays applied to characterize platelet inhibitors such as GpIIb–IIIa antagonists and various other antiplatelet agents. Furthermore, it is an established screening tool for the diagnosis of thrombocytopathies. Platelet aggregation testing can be performed with several techniques such as optical and electrical aggregometry and particle counting. There are several differences between these methods. Only particle counting and electrical aggregometry can be applied in whole blood studies, whereas for turbidimetric aggregation, samples of isolated platelet are required.

Kinetic information of the aggregation process can easily be obtained with electrical and turbidimetric aggregometry. However, in contrast to particle counting which is a discontinuous measurement, these two methods are only sensitive to late stages of platelet aggregation and are very insensitive to early stages of platelet aggregation.

Finally, it should be noted that aggregation studies have generally been performed with stirred platelet suspensions which have ill-defined flow regimes. The increasing use of microdevices with well-defined laminar flow can be expected to yield important information on shear-dependent platelet aggregation and effects of antithrombotic drugs.

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